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Translational Regulation of *oskar* mRNA by Bruno, an Ovarian RNA-Binding Protein, Is Essential

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Summary

Oskar (osk) protein directs the deployment of *nanos* (*nos*), the posterior body-patterning morphogen in Drosophila. To avoid inappropriate activation of *nos*, *osk* activity must appear only at the posterior pole of the oocyte, where the *osk* mRNA becomes localized during oogenesis. Here, we show that translation of *osk* mRNA is, and must be, repressed prior to its localization; absence of repression allows osk protein to accumulate throughout the oocyte, specifying posterior body patterning throughout the embryo. Translational repression is mediated by an ovarian protein, bruno, that binds specifically to bruno response elements (BREs), present in multiple copies in the *osk* mRNA 3′UTR. Addition of BREs to a heterologous mRNA renders it sensitive to translational repression in the ovary.

Introduction

Specification of cell fates in the developing embryo has long been thought to involve the action of localized cytoplasmic determinants (reviewed by Davidson, 1986). In such a model, molecules provided by the mother are positioned selectively within the egg, allowing them to be partitioned unequally among the cells arising during the early cleavage stages of embryogenesis. The particular fate adopted by an individual cell is then influenced by the presence or absence of the localized determinant. In recent years, a variety of molecules that act as localized determinants have been identified, making it possible to begin to investigate how they are selectively deployed within the egg.

One strategy used often in Drosophila melanogaster and Xenopus laevis relies on the prelocalization of mRNAs. While most maternal mRNAs eventually become dispersed throughout the oocyte cytoplasm, a few become concentrated at specific sites. For example, in Drosophila, the *oskar* (*osk*) and *nanos* (*nos*) mRNAs, required for posterior body patterning, are positioned at the posterior pole of the oocyte, while the *bicoid* (*bcd*) mRNA, required for anterior body patterning, is localized anteriorly. These localized mRNAs act as local sources for synthesis of the encoded proteins, which are themselves the localized determinants or spatial cues. The importance of mRNA localization in deploying cytoplasmic determinants in Drosophila has been highlighted by genetic analyses of body patterning. Among collections of mutants that specifically disrupt the basic coordinate system of the anteroposterior body plan, many prove to be defective in the localization of the *bcd*, *osk*, or *nos* mRNAs (reviewed by St Johnston and Nüsslein-Volhard, 1992).

Nevertheless, other mechanisms are likely to contribute to the proper deployment of body-patterning activities. The *bcd*, *osk*, and *nos* mRNAs are all synthesized outside of the oocyte, within the adjacent and interconnected nurse cells (St Johnston et al., 1989; Kim-Ha et al., 1991; Ephrussi et al., 1991; Wang et al., 1994). Localization takes many hours to complete, and the mRNAs accumulate at various sites before reaching their ultimate destinations. Thus, a potential difficulty arises: how is the activity of the determinants restricted prior to the localization of the encoding mRNAs? As the inappropriate spatial distribution of body-patterning determinants can reprogram the basic body plan with lethal consequences (for examples, see Frohnhöfer et al., 1986; Driever et al., 1990; Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992; Webster et al., 1994), restricting the activity of the determinants during the interval between mRNA synthesis and localization is likely to be important.

Translational regulation is one mechanism widely used to control the activities of maternal mRNAs (reviewed by Wickens, 1992). Many maternal mRNAs are repressed, or masked, during oogenesis and become translationally active only after fertilization or passage through another developmental checkpoint. A combination of mRNA localization and translational repression could bring about the desired distributions of proteins that function as cytoplasmic determinants. Indeed, recent results have indicated that localized mRNAs are subject to translational control in the normal course of development. Sallés et al. (1994) found that regulated polyadenylation underlies the translational activation of *bcd* mRNA early in embryogenesis. Elongation of poly(A) tails is a common mechanism by which maternal mRNAs are unmasked, and the *bcd* mRNA is probably one of a large group of Drosophila maternal mRNAs regulated in this fashion. The posteriorly localized *cyclin B* and *nos* mRNAs are also under translational control (Dalby and Glover, 1993; Gavis and Lehmann, 1994). For *nos*, at least, regulated polyadenylation is not involved (Sallés et al., 1994). Instead, translation appears to depend on posterior localization; in *osk* mutants, localization does not occur, and nos protein does not accumulate (Gavis and Lehmann, 1994). Nevertheless, it remains unclear whether translational control is essential for the correct distribution of these body-patterning activities, or whether mRNA localization alone is sufficient.

Here, we describe the translational regulation of the *osk* mRNA and the consequences of its failure. The *osk* gene directs deployment of *nos*, the posterior body-patterning determinant, at the posterior pole of the embryo. If *osk* activity is not confined to the posterior pole, *nos* is activated ectopically, and posterior body pattern elements are

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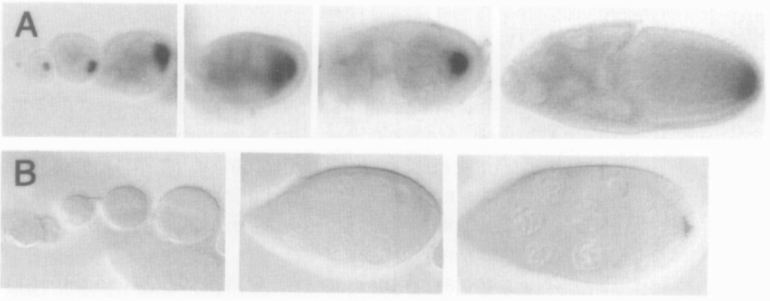

Figure 1. *osk* mRNA and Protein Distributions during Oogenesis

osk mRNA and proteins were detected in whole-mount ovary preparations by in situ hybridization and immunohistochemistry, respectively. Egg chambers of increasing age are displayed from left to right, with the posterior of the oocyte at the extreme right of each egg chamber. *osk* mRNA is initially concentrated in the oocyte (seen clearly in the left panel), where it eventually becomes localized to the posterior pole (central and right) and persists at that site into embryogenesis. This posterior localization appears to be a prerequisite for the appearance of osk protein, as seen in (B). In the early stages of oogenesis, no osk protein is detected (left panel), despite the high local concentration of *osk* mRNA in the oocyte. As oogenesis proceeds, there is a dramatic appearance of osk protein, only at the posterior pole, during stage 9; the stage 8 egg chamber in the center panel has no detectable osk protein, while the stage 9 egg chamber in the right panel has a prominent and tightly localized zone of osk protein at the posterior pole. This pattern persists into embryogenesis.

expanded at the expense of anterior structures (Ephrussi and Lehmann, 1992; Smith et al., 1992; Webster et al., 1994). Through the identification and characterization of bruno, an ovarian protein that binds specifically to multiple sites in *osk* mRNA, we find that translation of *osk* mRNA is normally repressed prior to its localization at the posterior pole of the oocyte. When bruno binding is eliminated, *osk* mRNA localization remains normal, but osk protein appears precociously throughout the oocyte, and *nos* is activated throughout the embryo. Thus, both mRNA localization and translational control are essential for the correct deployment of *osk* activity.

Results

osk mRNA is transcribed early in oogenesis, when it is localized to the oocyte (Kim-Ha et al., 1991; Ephrussi et al., 1991). At later stages, the distribution of *osk* mRNA undergoes major changes. In the oocyte, *osk* mRNA accumulates transiently at the anterior margin and then becomes concentrated at the posterior pole, where it persists for the remainder of oogenesis and during the cleavage stages of embryogenesis (Kim-Ha et al., 1991; Ephrussi et al., 1991; Figure 1A). Despite the presence of high levels of *osk* mRNA throughout oogenesis, osk protein is detected only after localization of the mRNA to the posterior pole of the oocyte (Figure 1B). Thus, at earlier stages, *osk* mRNA must be translationally inactive, or the osk protein must be unstable. If translation of *osk* mRNA is indeed regulated, control is likely to be mediated by sequences in the *osk* mRNA to which factors bind. However, none of the genes known to act in provision or control of *osk* activity have been shown to encode proteins that bind specifically to *osk* mRNA. A biochemical approach is therefore attractive for identifying such factors.

An Ovarian Protein Binds Specifically to the *osk* mRNA 3′ Untranslated Region

An ultraviolet (UV) cross-linking assay was used to detect ovarian proteins that bind to the *osk* mRNA (Figure 2). One readily detectable protein with an apparent molecular weight of 80 kDa binds specifically to the *osk* mRNA 3′ untranslated region (3′UTR); we call this protein bruno. Three distinct segments, termed the A, B, and C regions, contain bruno-binding sites (Figures 2A and 2B). The A and B regions are adjacent to one another (and can therefore be transcribed as a single unit to form the AB region) near the beginning of the 3′UTR, whereas the C region is located downstream of the AB region, close to the polyadenylation site.

The binding of bruno to several discrete regions of the 3′UTR suggests that the interaction is specific. To confirm the specificity of binding, and to demonstrate that all three regions are indeed bound by the same protein, we assayed the ability of several RNAs to compete for bruno binding to either the AB or the C region. Addition of excess unlabeled AB region transcripts efficiently competed with either the AB (Figure 2C) or the C region probe (data not shown) for bruno binding. Similarly, A or B region competitor RNAs could also compete for bruno binding, although less efficiently than the AB region competitor (Figure 2C). Conversely, nonspecific competitors, including transcripts from another part of the *osk* 3′UTR and transcripts from the *bcd* 3′UTR, did not efficiently compete for bruno binding. We conclude that bruno binds specifically to multiple discrete sites in the *osk* mRNA.

Identification of the Bruno-Binding Sites

To define the bruno-binding sites better, the C region was examined in greater detail (Figure 3). Although bruno could bind each of three progressive 5′ deletion transcripts, bruno binding to the shortest of these transcripts was reduced (Figure 3B), suggesting that the CΔ3 deletion removes or interferes with a binding site. Deletion of internal sequences, in CΔ4, completely abolished bruno binding (Figure 3B), identifying a region essential for binding.

To investigate whether a sequence motif was present in all bruno-binding regions, and thus a candidate for the binding site, the portion of the C region required for binding was compared with the A and B regions. A 7–9 nt sequence is repeated twice in the C region and also appears in the A and B regions (Figure 3C, legend). The core sequence is U(G/A)U(A/G)U(G/A)U, with an additional U residue present at either or both of the 5′ and 3′ ends. The importance of this motif was addressed by introducing subtle mutations into either or both copies from the C region.

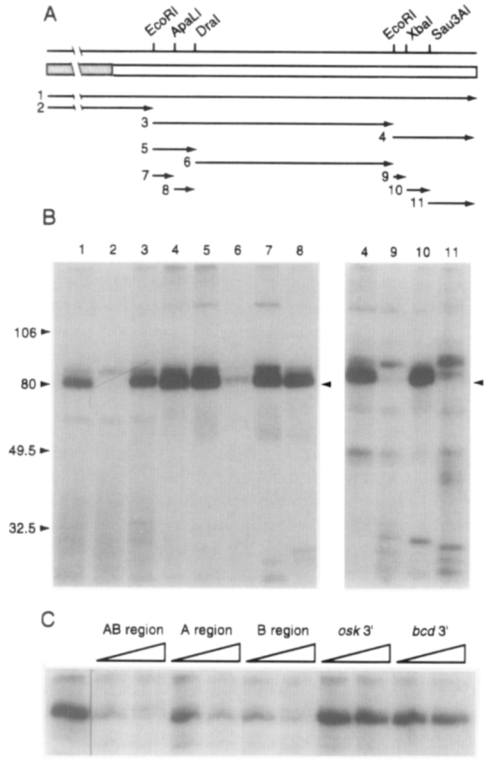

Figure 2. An 80 kDa Ovarian Protein Binds to Multiple Sites in the *osk* mRNA 3′UTR

(A) An *osk* cDNA is diagrammed schematically (top); the coding region and 1043 nt 3′UTR are shown as stippled and open boxes, respectively. A partial restriction map of the 3′UTR, showing sites used in subcloning, is shown above. Transcripts used as probes for the UV cross-linking assays in (B) are identified by numbers and shown below.

(B) Proteins UV cross-linked to labeled RNA probes are displayed by SDS–PAGE and autoradiography. The various lanes correspond to separate binding experiments, in which equal amounts of different RNA probes, labeled as in (A), were used. A prominent protein of about 80 kDa (bruno), indicated by an arrowhead at the right of each panel, binds strongly to three regions that are most narrowly defined by transcripts 7, 8, and 10. These portions of the *osk* 3′UTR are subsequently referred to as the A, B, and C regions. Because the A and B regions are contiguous, they are collectively called the AB region. Binding to the remaining parts of the *osk* 3′UTR is either substantially weaker or not detected.

(C) Competition binding experiments. Binding of bruno to labeled AB region RNA (transcript 5 in [A]) was performed in the presence of unlabeled competitor RNAs. The left lane shows the binding in the absence of competitor RNA. Each additional pair of binding reactions contained 100- and 300-fold excesses of the unlabeled competitor RNAs. The AB, A, and B region RNAs are as defined above (RNAs 5, 7, and 8, respectively, from [A]). The *osk* 3′ RNA corresponds to RNA 6 in (A), and the *bcd* 3′ RNA consists of much of the *bcd* 3′UTR (nucleotides 4100–4883; Berleth et al., 1988).

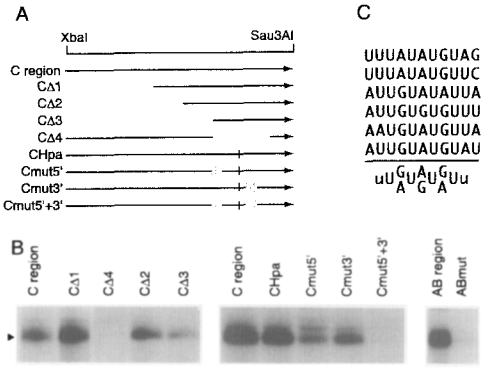

Figure 3. Definition of Bruno-Binding Sites

(A) Schematic diagram of the C region RNAs used in binding assays. The 71 nt C region is shown as a horizontal line, with 5′ to the left. Deletions are indicated by gaps. A single nucleotide change introduced to create a HpaI restriction site (to simplify cloning) is indicated by a vertical line. In Cmut5′, UGUAUGU is replaced by UUUGAGU; in Cmut3′, UGUAUGU is replaced by UUGAGUU; these changes are shown as shaded boxes.

(B) UV cross-linking assays. Equal amounts of radiolabled RNAs from (A) were used as probes in separate binding assays. The right panel shows the results of binding assays with the AB region probe, or the same segment with the bruno consensus sequences mutated (ABmut; see text).

(C) Sequences from the region missing in the CΔ4 mutant were compared with the A and B region sequences, revealing a common sequence motif that is presented at the bottom. *osk* sequences are shown above the horizontal line: the first two copies are from the A region, the next two from the B region, and the last two from the C region. Mutations within the consensus sequence clearly interfere with bruno binding (as seen in [B]), but we do not yet know what constitutes a minimal binding site. Sequences that fit this consensus but contain only A at the purine positions (as found in the *nos* mRNA 3′UTR, for example) either fail to bind bruno in vitro or bind very weakly (data not shown).

Transcripts containing mutations in only one copy of the shared sequence (Cmut5′ or Cmut3′) displayed reduced bruno binding, while mutating both copies (Cmut5′+3′) completely abolished bruno binding (Figure 3B). Thus, the observed consensus sequence (or some feature thereof) does appear to be required for bruno binding to the C region. To investigate whether the same sequence is also important for binding of bruno to the AB region, all copies of this motif in the A and B regions were mutated (ABmut; see Experimental Procedures). Binding of bruno to the mutant transcript was greatly reduced (Figure 3B), confirming the importance of the consensus sequence. We call the bruno-binding sites bruno response elements, or BREs.

Using *oskBRE*$^-$ Mutants to Test the Role of Bruno

Mutant flies lacking bruno activity have not to our knowledge been identified. Nevertheless, the role of bruno in *osk* function can be addressed by creating *osk* transgenes lacking the bruno-binding sites. The ABmut and Cmut5′+3′ mutations, which substantially reduce bruno binding in vitro (Figure 3), were introduced into an otherwise wild-type *osk* gene; the mutated gene is called *oskBRE*$^-$, and its transcripts are expected to bind only poorly to bruno in vivo. Flies expressing the P[*oskBRE*$^-$] transgene or a control P[*osk*$^+$] transgene (Smith et al., 1992) were established by P element–mediated transformation. In the following sections, maternal genotypes are used to describe the resulting embryos, as only maternal-effect mutants or transgenes are discussed.

osk is required for posterior body patterning. To investigate whether the P[*osk*$^+$] and P[*oskBRE*$^-$] transgenes differ in their abilities to provide *osk* function, a rescue assay was used. Flies homozygous for an *osk* mutation and car-

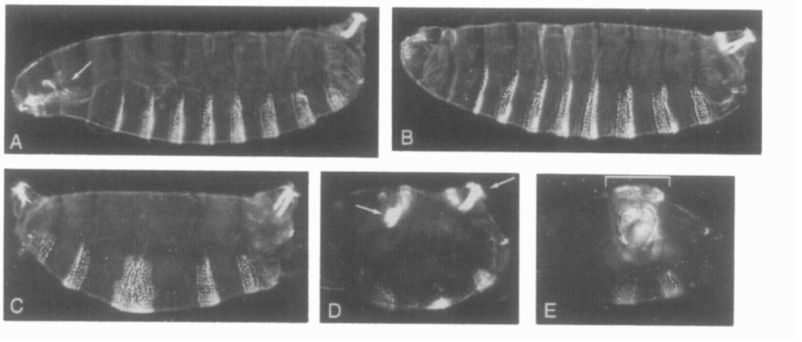

Figure 4. Body Patterning Phenotypes Due to Lack of Bruno-Binding Sites

Cuticles of embryos from mothers homozygous for *osk*^{6} and carrying either the control P[*osk*$^{+}$] (A) or the P[*oskBRE*$^{-}$] transgene (B–E) are shown.

(A) The wild-type cuticlar pattern displayed by almost all embryos from *osk*^{6} flies carrying either one or two copies of the P[*osk*$^{+}$] transgene.

(B) An embryo with anterior pattern deletions. Head structures, indicated with an arrow in (A), and the first thoracic denticle belt are missing. Most embryos (88%) from *osk*^{6} flies carrying one copy of the P[*oskBRE*$^{-}$] transgene have anterior pattern deletions, which vary in extent and result from ectopic *nos* activity (Wharton and Struhl, 1991). Of the remaining embryos, 7% have wild-type cuticles, while 5% display the bicaudal phenotype (C). The distribution of embryos between these classes is given for the P[*oskBRE*$^{-}$]-7 transgene (see Experimental Procedures). When two copies of the P[*oskBRE*$^{-}$] transgene are present in *osk*^{6} mothers, the posteriorization of the body pattern is more severe. Now 13% of the embryos have the bicaudal phenotype shown in (C), 4% have less severe defects, and the majority (83%) display more severe posteriorized phenotypes (D and E). The embryo in (D) retains only one and a half duplicated abdominal segments, and the refractile filzkörper (arrows) remain stretched and positioned near the termini of the embryo. The more severely posteriorized embryo in (E) has only a single duplicated abdominal segment. The filzkörper are no longer recognizable; these and other terminal structures (bracketed) now occupy a central position along the anteroposterior body axis. The severity of this phenotype is unexpected; embryos lacking *bcd* and *hunchback*, the genes known to be negatively regulated by *nos*, have weaker phenotypes (Hülskamp et al., 1990). Nevertheless, *nos* is required to express the *oskBRE*$^{-}$ phenotype (Table 1), suggesting that additional targets for *nos* action remain to be identified.

rying one or two copies of either the P[*oskBRE*$^{-}$] or P[*osk*$^{+}$] transgene were allowed to lay eggs, and the cuticles of the embryos were examined. The P[*osk*$^{+}$] transgene provides normal *osk* activity (Smith et al., 1992; Figure 4A), and the P[*osk*$^{+}$]; *osk*$^{-}$ embryos have wild-type cuticles. In contrast, the P[*oskBRE*$^{-}$]; *osk*$^{-}$ embryos display substantial patterning defects; a single copy of the transgene fully restores posterior body patterning, but also causes anterior pattern deletions and sometimes results in bicaudal embryos in which anterior structures are replaced by a mirror-image duplication of posterior structures (Figures 4B and 4C). These defects indicate a posteriorization of the embryo and can be attributed to excess or mislocalized *osk* activity (Ephrussi and Lehmann, 1992; Smith et al., 1992; Webster et al., 1994). The shift to posterior body patterning is enhanced when two copies of P[*oskBRE*$^{-}$] are present; some embryos are bicaudal, while the majority display an extreme posteriorized phenotype (Figures 4D and 4E).

Molecular Defects Associated with the *oskBRE*$^{-}$ Phenotype

The *oskBRE*$^{-}$ phenotype suggests that bruno normally acts to restrict *osk* activity. Given the relative distributions of *osk* mRNA and protein during oogenesis (see Figure 1), it seems likely that bruno is involved in either *osk* mRNA localization or translational control. Monitoring the distribution of sequence-tagged transgene mRNAs, we find that the *BRE*$^{-}$ mutations have no effect on *osk* mRNA localization (data not shown). In contrast, *osk* mRNA translation is affected. Normally, osk protein appears only after *osk* mRNA is localized to the posterior pole of the oocyte in stages 8–9 of oogenesis (see Figures 1 and 5A). However, flies carrying the P[*oskBRE*$^{-}$] transgene express osk protein precociously. Immunostaining with osk antibodies reveals osk protein dispersed throughout the oocyte cytoplasm during stages 7–8 (Figure 5B), mirroring the distribution of *osk* mRNA. As oogenesis proceeds, the distribution of osk protein continues to parallel that of the *osk* mRNA, becoming restricted to the posterior pole. Thus, the wild-type pattern of osk protein distribution is restored during the later stages of oogenesis; we do not know whether the osk protein that accumulates prematurely is simply diluted by expansion of the oocyte or whether it is degraded. The amount of posteriorly localized osk protein does not appear to differ significantly between flies carrying the P[*oskBRE*$^{-}$] transgene and flies carrying the P[*osk*$^{+}$] transgene. We conclude that bruno is required for preventing translation of *osk* mRNA prior to its localization at the posterior pole of the oocyte.

BREs Confer Translational Repression on a Heterologous mRNA

To investigate whether bruno-dependent translational repression can be conferred on a heterologous mRNA, we used the *exuperantia* (*exu*) gene, which has two features that make it attractive for such an approach. First, the *exu* mRNA is present and translationally active in the appropriate tissue, the germline cells of the ovary (Macdonald et al., 1991; Marcey et al., 1991), and should therefore have the opportunity to interact with bruno. Second, we had previously found that certain foreign sequences could be inserted into the *exu* 3′UTR with no effect on *exu* function (P. M. M., unpublished data). Transgenic flies expressing either the wild-type *exu* gene (P[*exu*$^{+}$]) or an *exu* gene modified by addition of *BRE*$^{+}$ sequences in the 3′UTR (P[*exu*$^{+}$/*BRE*$^{+}$]) were established and introduced into an *exu*$^{-}$ background. Although both types of transgenes are transcribed at similar levels (Figure 6A), the amount of exu protein expressed from the P[*exu*$^{+}$/*BRE*$^{+}$] transcript is reduced (Figure 6B), suggesting that addition of *BRE*$^{+}$ sequences confers translational repression on the *exu* mRNA.

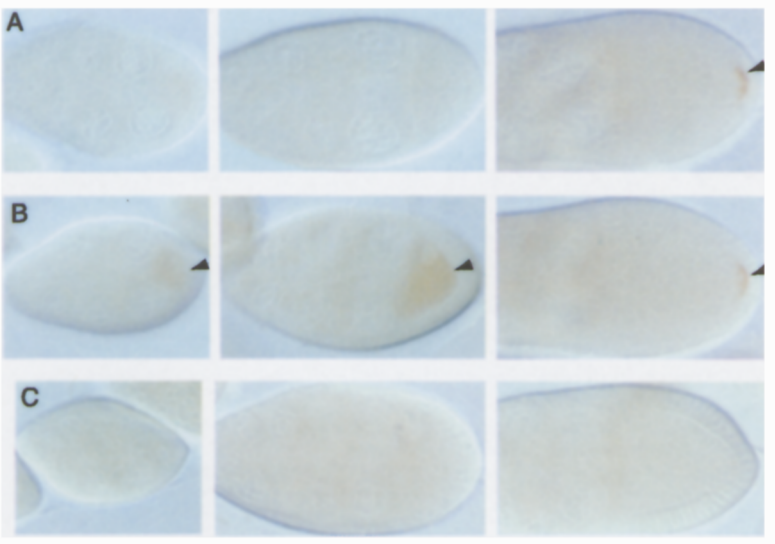

Figure 5. Osk Protein Appears Prematurely When Bruno-Binding Sites Are Absent

Ovaries of flies carrying a P[*osk*$^+$] transgene (A) or a P[*oskBRE*$^-$] transgene (B and C) were tested for the presence of osk protein by immunostaining. Each row shows egg chambers of increasing maturity. The osk protein appears brown in the immunohistochemical staining and is highlighted by arrowheads.

(A) In ovaries expressing the P[*osk*$^+$] transgene, as in wild type, no osk protein is detected prior to localization of the *osk* mRNA at the posterior pole of the oocyte.

(B) In contrast, the P[*oskBRE*$^-$] transgene directs precocious accumulation of osk protein, which appears throughout the oocyte in stages 7 and 8 of oogenesis (left and center). As oogenesis progresses, this uniform staining disappears, such that the normal distribution of osk protein is restored (right).

(C) In *stau* mutants carrying the P[*oskBRE*$^-$] transgene, no osk protein is detected at any stage.

oskBRE$^-$ Phenotypes in Posterior Group Mutants

The posterior group genes, including *osk*, are required maternally for providing the *nos* morphogen, which directs posterior body patterning in the embryo. Mutants of five of the posterior group genes, *cappuccino* (*capu*), *spire* (*spir*), *mago nashi* (*mago*), *staufen* (*stau*), and *oo18 RNA-binding* (*orb*), fail to localize *osk* mRNA to the posterior pole of the oocyte (Kim-Ha et al., 1991; Ephrussi et al., 1991; Newmark and Boswell, 1994; Christerson and McKearin, 1994). Two observations, taken together, suggest that these genes could also be required for the translation of *osk* mRNA. First, the simple appearance of osk protein seems sufficient to direct *nos* activation (see Figure 5; Ephrussi and Lehmann, 1992; Webster et al., 1994). Second, mutants of the five genes required for *osk* mRNA localization lack posterior body-patterning activity. Thus, it is likely that osk protein is not made by these mutants, suggesting that localization of *osk* mRNA is normally a prerequisite for the provision of *osk* activity. To learn more about the possible roles of these five genes in translational control of *osk* mRNA, we investigated which are required for provision of *osk* activity when bruno-dependent translational repression is lacking. This was accomplished by crossing the P[*oskBRE*$^-$] transgene into the appropriate mutant backgrounds and examining cuticular phenotypes of embryos from mutant mothers. By this test, the posterior group genes required for localization of *osk* mRNA fall into two classes (Table 1). Flies homozygously mutant for *capu*, *spir*, *mago*, or *orb* all produce embryos that lack abdominal segments (Manseau and Schüpbach, 1989; Boswell et al., 1991; Christerson and McKearin, 1994), but addition of one copy of the P[*oskBRE*$^-$] transgene restores abdominal segmentation and also interferes with anterior body patterning to varying extents in the different mutants. In contrast, flies homozygously mutant for *stau* produce embryos that lack abdominal segments, even if the P[*oskBRE*$^-$] transgene is present. These results indicate that *stau* alone remains required for *osk* activity when bruno-mediated translational repression is missing, suggesting that *stau* activates *osk* mRNA translation. We confirmed that the precocious translation of osk protein by the P[*oskBRE*$^-$] transgene was indeed eliminated in *stau* mutant ovaries (see Figure 5).

The phenotype of the P[*oskBRE*$^-$] transgene was also tested in two other genetic backgrounds: *nos*$^-$ (Lehmann and Nüsslein-Volhard, 1991) and wild type. Flies homozy-

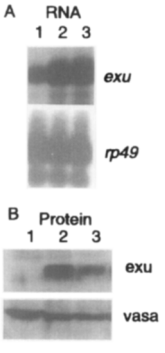

Figure 6. BRE Sequences Confer Translational Repression on a Heterologous mRNA

The flies used for analysis were the following: lane 1, *exu*^{4}/*exu*^{4}; lane 2, *exu*^{4}/*exu*^{4}; P[*exu*$^+$]/TM2; lane 3, *exu*^{4}/*exu*^{4}; P[*exu*$^+$/*BRE*$^+$]/TM2.

(A) Endogenous and transgene mRNAs were detected by an RNase protection assay. An *rp49* control was used to ensure that similar amounts of total RNA were used for each genotype. The *exu*^{4} mutant accumulates less *exu* mRNA than wild type (Hazelrigg et al., 1990), and so most of the RNA detected in lanes 2 and 3 is from the transgenes; similar amounts are detected.

(B) Exu proteins were detected by Western blot analysis. The vas protein control was used to ensure that similar amounts of total protein were used for each genotype. Because the *exu*^{4} mutant makes no detectable antigen (Macdonald et al., 1991; Marcey et al., 1991; lane 1), all of the exu protein detected in lanes 2 and 3 is made from the transgene mRNAs. Substantially more exu protein is found in the P[*exu*$^+$] ovaries than in the P[*exu*$^+$/*BRE*$^+$] ovaries. Thus, the P[*exu*$^+$/*BRE*$^+$] mRNA is translationally repressed, although not with high efficiency.

Table 1. Embryonic Phenotypes Resulting from P[*oskBRE*$^-$] Expression in Various Genetic Backgrounds

Background	No Abdomen (%)	Wild Type (%)	Anterior Deletions (%)	Bicaudal (%)
capu^{-a}			30	68
spir^{-a}			10	89
mago$^-$		28	35	37
orb$^-$		41	30	28
stau$^-$	94			4[b]
nos$^-$	100[c]			
osk$^-$		7	88	5
osk$^-$/+[d]		15	75	10
Wild type		87	13	

Maternal genotypes are given (see Experimental Procedures). Embryos that failed to differentiate a cuticle were not included in the analysis.
[a] We confirmed by in situ hybridization that the acquisition of posterior body patterning activity was achieved without restoring posterior localization of *osk* mRNA.
[b] The rare bicaudals are consistent with observations suggesting that the requirement for *stau* in *osk* translation is not absolute. See Discussion.
[c] The same phenotype was obtained when two copies of the transgene were introduced into *nos*$^-$ flies.
[d] Data are shown for *osk*^{6}. Similar results were obtained using *osk*^{9} (a nonsense mutant) or *Df(3R)p*^{XT103}, an *osk* deletion.

gously mutant for *nos* lack abdominal segments, independent of the presence or absence of the P[*oskBRE*$^-$] transgene. This is the expected result if the pattern defects caused by the P[*oskBRE*$^-$] transgene are mediated by *nos*, the posterior body-patterning morphogen. To demonstrate this point more conclusively, we also tested the consequences of expressing two copies of the P[*oskBRE*$^-$] transgene in *nos* mutant flies; again, the *nos*$^-$ phenotype was observed (Table 1).

Surprisingly, the phenotypes caused by expression of a single copy of the P[*oskBRE*$^-$] transgene in an *osk* mutant background are largely suppressed by restoring both wild-type copies of the *osk* gene (Table 1). While >90% of embryos from P[*oskBRE*$^-$]; *osk*$^-$ mothers display excessive posterior body-patterning activity (in the form of anterior pattern deletions; see Figure 4), <20% of embryos from P[*oskBRE*$^-$]; *osk*$^+$ mothers have such defects. Suppression of the P[*oskBRE*$^-$] phenotype does not occur in *osk* heterozygotes. Thus, suppression depends not on the simple presence of the *osk*$^+$ gene, but rather on the amount of the *osk*$^+$ gene product. The implications of these results are considered in the Discussion.

Discussion

Many maternal mRNAs are subject to translational control, facilitating the stockpiling of huge quantities of translationally inert mRNAs in the oocyte. When translationally activated, these mRNAs will support early development of the embryo, allowing rapid nuclear division cycles in which transcription is necessarily limited. In a few cases, translational control of maternal mRNAs has been shown to play a more instructive role. For example, local repression of *hunchback* mRNA translation in the Drosophila embryo is crucial for proper posterior body patterning (Wharton and Struhl, 1991). With the discovery of localized maternal mRNAs, attention has turned to questions of whether they too are translationally controlled, and more importantly, whether such control is necessary for the development of the animal. Among the localized mRNAs of Drosophila, differences in mRNA and protein distributions suggested that translational control occurred (for example, see Driever and Nüsslein-Volhard, 1988a). More direct evidence of such control was first provided for the posteriorly localized *cyclin B* mRNA (Dalby and Glover, 1993). Subsequently, evidence demonstrating translational control has been provided for the *nos* (Gavis and Lehmann, 1994) and *bcd* (Sallés et al., 1994) mRNAs. Because some of the localized mRNAs encode proteins that direct specific programs of body patterning, it has been attractive to speculate that translation and localization are coordinated, and that translation must be prevented before the mRNAs are appropriately localized, thus ensuring that the patterning proteins are deployed only where desired. However, while correlations have been made between mRNA localization and translation (Gavis and Lehmann, 1994), there has been to our knowledge no experimental evidence to determine whether translational control is crucial for restricting the activities of the patterning proteins, or whether mRNA localization by itself is sufficient.

We have shown here that *osk* mRNA, too, is subject to translational control. Direct evidence for translational repression was provided by our demonstration that mutations in the *osk* mRNA 3'UTR (which should have no effect on osk protein stability) induced the precocious appearance of osk protein; such mutations make the patterns of *osk* mRNA and protein expression more closely similar than in wild type, although still not identical. Furthermore, we have shown that translational repression of *osk* mRNA is essential for restricting the distribution of osk protein, thereby preventing the ectopic activation of the *nos* posterior body-patterning morphogen. Our results now demonstrate that mRNA localization alone is not sufficient for dictating the distributions of spatial patterning molecules, and they provide direct experimental support for the notion that translational control of localized mRNAs has functional importance.

Relationships between *osk* mRNA Localization and Translational Regulation

We now know of two levels at which posttranscriptional

control of *osk* expression is exerted: mRNA localization and translation. Three levels of *osk* translational control can be considered. One is the repression of *osk* mRNA translation that occurs prior to, and is independent of, posterior localization. Release from this repression is a second level of translational control. It appears to require the posterior localization of *osk* mRNA, as mutants defective in localization, and normally lacking *osk* activity, regain *osk* activity (but not *osk* mRNA localization) when the initial repression is abolished (Table 1). This step therefore involves mRNA localization factors and presumably a factor or event brought into play by positioning *osk* mRNA at the posterior pole of the oocyte. The third level of control is a translational activation event that is independent of both localization and the initial repression and is suggested by the phenotype of *stau* mutants. We now discuss in more detail the roles played by known proteins and genes.

Repression of *osk* mRNA translation is mediated by bruno, an ovarian protein that binds to multiple sites in the *osk* mRNA 3′UTR. For *osk* mRNA, the function of bruno appears to be restricted to translational control, as mRNA localization is not impaired by loss of bruno binding. Bruno does not appear to be encoded by any of the posterior group genes, as extracts from these (and other) mutants (*Bicaudal C* [*BicC*], *Bicaudal D* [*BicD*], *capu*, *egalitarian* [*egl*], *mago*, *nos*, *osk*, *orb*, *pumilio* [*pum*], *spir*, *stau*, *tudor* [*tud*], *vasa* [*vas*], and *valois* [*vls*]) retain the RNA-binding activity of bruno (data not shown). A better understanding of how bruno represses translation will likely come from its purification and from the development of in vitro systems to study its function. In addition, it should be instructive to investigate whether bruno binds to and controls expression of other mRNAs. In preliminary experiments, we have found that *gurken* mRNA (Neuman-Silberberg and Schüpbach, 1993) is bound by bruno, and many other ovarian mRNAs have potential BREs (data not shown).

In wild-type ovaries, osk protein begins to accumulate when *osk* mRNA arrives at the posterior pole of the oocyte. Because we now know that translational repression prevents the earlier accumulation of osk protein, we can infer that posterior localization of the mRNA is accompanied by release from bruno-dependent translational repression. Thus, under normal circumstances, posterior localization appears to be a prerequisite for the translation of *osk* mRNA. This notion is supported by the behavior of mutants of several genes known to be required for localization of *osk* mRNA. In flies homozygous for mutations in the *capu*, *spir*, *mago*, or *orb* genes, *osk* mRNA is not localized (and presumably not translated), and there is no detectable *nos* activity (progeny embryos lack abdominal structures). However, if translational repression by bruno is abolished (with the P[*oskBRE*$^-$] transgene), then all four mutants regain substantial amounts of *nos* activity.

A further form of translational activation of *osk* mRNA is suggested by analysis of the *stau* mutant. Like the *capu*, *spir*, *mago*, and *orb* genes, *stau* is required for *osk* mRNA localization and thus helps fulfill the requirement of posterior mRNA localization for translation. Unlike mutants in the other four genes, however, *stau* mutants still lack *osk* activity when bruno-dependent translational repression is abolished. Indeed, the osk protein that accumulates uniformly throughout the oocytes of P[*oskBRE*$^-$] transgenic ovaries disappears in *stau* mutants. This result has two implications: first, that *stau* is required for *osk* translation independent of localization, and second, that release of *osk* mRNA from the bruno-dependent repression is not the role played by *stau*. Our evidence that *stau* is a translational regulator is not direct, as we have not identified RNA elements in the *osk* mRNA that bind stau protein and are required for *stau*-dependent translation. Nevertheless, translational activation is a more likely mechanism than the alternative, stabilization of osk protein, as stau protein contains multiple copies of a double-stranded RNA–binding motif and appears to function by binding to mRNAs (St Johnston et al., 1992; Ferrandon et al., 1994). Although *stau* is conventionally portrayed as acting in mRNA localization events, since both *bcd* mRNA and *osk* mRNA are delocalized in *stau* mutants (reviewed by St Johnston and Nüsslein-Volhard, 1992), the possibility of a more direct role in translation is consistent with other lines of work. The anterior pattern defects caused by *stau* mutants suggest that *bcd* activity is reduced, as expected for inefficient translation, rather than being dispersed, as expected for inefficient anterior localization (Driever and Nüsslein-Volhard, 1988b). Furthermore, the degree of *bcd* mRNA delocalization observed in *stau* mutants can be mimicked by mutations in the *bcd* 3′UTR, yet these *bcd* mutants do not interfere with anterior body patterning (Macdonald et al., 1993). Thus, the *bcd* mRNA localization defects in *stau* mutants cannot account for their anterior pattern defects.

Two requirements for *osk* translation, posterior mRNA localization and *stau* activity, are not absolute. The *capu*, *spir*, and *stau* genes, all required for *osk* mRNA localization, become at least partially dispensable for *osk* activity when *osk* is overexpressed by raising the *osk* gene dosage (Smith et al., 1992). Furthermore, substantial amounts of *osk* activity appear in double-mutant combinations of *BicD* (which causes a transient anterior mislocalization of *osk* mRNA in the oocyte [Kim-Ha et al., 1991; Ephrussi et al., 1991]) with either *capu*, *spir*, or *stau* mutants (Manseau and Schüpbach, 1989; Lehmann and Nüsslein-Volhard, 1991). Thus, the on or off state of *osk* translation appears to reflect the contributions of multiple factors, some of which can be made dispensable under certain conditions.

Role of *osk* in Activation of *nos*

The requirement for *osk* in activation of *nos* is well established (reviewed by St Johnston and Nüsslein-Volhard, 1992). It has been less clear what, if any, restrictions limit the ability of *osk* to initiate that process. For example, would a low level of osk protein throughout the oocyte cytoplasm activate *nos* uniformly, or must osk protein be concentrated to be active, perhaps in close association with the cortex? Analysis of the action of the Drosophila virilis *osk* gene, when expressed in D. melanogaster, suggested that osk protein could act anywhere in the oocyte (Webster et al., 1994). Our current results now reinforce that conclusion, using the native D. melanogaster osk protein. The only molecular defect arising from lack of bruno binding to *osk* mRNA is the appearance of osk protein

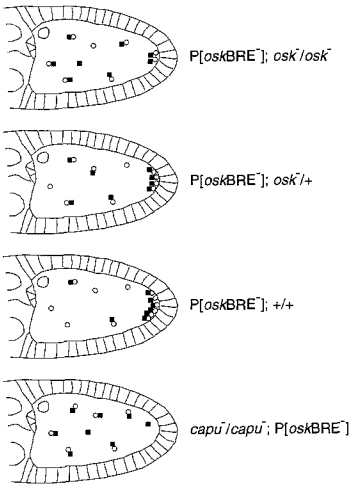

Figure 7. A Speculative Model for Osk Protein Interactions in the Oocyte

Each cartoon shows the oocyte of a stage 10 egg chamber, oriented with the posterior to the right. Open circles represent osk protein, either localized at the posterior pole or dispersed in the ooplasm. Closed squares represent a factor that interacts with osk protein and is required for *nos* activation; only joined circles and squares activate *nos*. The factor is efficiently recruited to the posterior pole by localized osk protein and is not in great excess. Consequently, changes in the amount of localized osk protein, as found in the different genotypes, influence the activity of the unlocalized osk protein.

throughout the oocyte during stages 7–8 of oogenesis. Although this is a relatively modest and apparently transient change in the overall pattern of osk protein accumulation, it leads to a dramatic ectopic activation of the *nos* morphogen and severe body patterning defects. We can detect no local ectopic concentration of nos protein in the resulting embryos (data not shown), consistent with the notion that *nos* has been activated throughout the cytoplasm. These results support a model for *osk* action in which osk protein by itself can initiate the assembly of ribonucleoprotein complexes that are competent for the translation of *nos* mRNA. If this is indeed how osk protein acts, it represents a potential danger to the animal, since low levels of inadvertent misexpression of *osk* could activate *nos* inappropriately. However, a possible fail-safe mechanism is suggested by a curious feature of the P[*oskBRE⁻*] transgene: the phenotypes that it causes in an *osk* mutant background are partially suppressed by restoring both copies of the wild-type *osk* gene. One explanation of this phenomenon rests on two premises: that osk protein recruits factors involved in *nos* activation, and that one or more of these factors may not be present in great excess, opening the possibility of a competition between different pools of osk protein for limiting components. Both premises have experimental support (Hay et al., 1990; Lasko and Ashburner, 1990; Smith et al., 1992; J. Wilson and P. M. M., unpublished data). We suggest that a disparity in the abilities of the localized and unlocalized osk proteins to recruit these factors can account for the suppression of the P[*oskBRE⁻*] phenotype by wild-type *osk*. In this model (Figure 7), the degree of ectopic *nos* activation directed by the P[*oskBRE⁻*] transgene is dictated by the relative levels of unlocalized and posteriorly localized osk protein (open circles in Figure 7). In *osk* mutant flies, the only posterior osk protein is provided by the transgene, and the ratio of unlocalized to localized osk protein is high. This ratio is reduced somewhat by restoring one copy of *osk*$^+$, and to a greater degree by restoring two copies of *osk*$^+$. We speculate that with two copies of *osk*$^+$, the limiting factors (closed squares in Figure 7) are sufficiently depleted from the ooplasm that the unlocalized osk protein activates *nos* at a reduced efficiency. Why might the localized osk proteins be a better competitor for the limiting factors? Localized osk protein is highly concentrated, and it is associated with the egg cortex, two features that might promote its activity. This model also accounts for the high levels of ectopic *nos* activity found when the P[*oskBRE⁻*] transgene is expressed in flies mutant for the *capu*, *spir*, *mago*, or *orb* genes (Table 1). Each mutant is defective in *osk* mRNA localization, and no posteriorly localized osk protein is expected to be made. Thus, the unlocalized osk protein should have no competitors for *nos* activation.

Experimental Procedures

Preparation of Ovary Extracts

Ovaries of healthy, well-fed 3- to 4-day-old w^{1118} adult females were dissected in phosphate-buffered saline (PBS). After removal of PBS, ice-cold lysis buffer (150 mM NaCl, 1.0% Nonidet P-40 [NP-40], 50 mM Tris–HCl [pH 8.0]) was added (2.5 μl per ovary pair), and the ovaries were homogenized with a plastic pestle in a 1.5 ml microfuge tube. The lysate was cleared by centrifugation at 10,000 rpm for 10 min at 4°C, an equal volume of 40% glycerol was added to the supernatant, and the extract was stored at −70°C. Some extracts were prepared with a lysis buffer consisting of 150 mM NaCl, 1.0% NP-40, 50 mM HEPES–OH (pH 7.9), 1 mM phenylmethylsulfonyl fluoride (PMSF) with no change in the results.

UV Cross-Linking Assay

UV cross-linking assays were performed much as described (Hedley and Maniatis, 1991). Protein extract equivalent to one pair of ovaries (~50 μg of protein) was preincubated with 10 μg of yeast tRNA in a 10 μl reaction mix containing 1 μl of 10× reaction buffer A (32 mM $MgCl_2$, 20 mM ATP, 200 mM creatine phosphate, 60 mM HEPES–OH [pH 7.9]), or 1 μl of 10× reaction buffer B (20 mM $MgCl_2$, 60 mM HEPES–OH [pH 7.9], 300 mM KCl) and 1 μl of 10 mg/ml heparin for 10 min at 30°C. A ^{32}P-labeled RNA probe (1 × 10^6 cpm) was added, and the sample was further incubated for 10 min at 30°C. The sample was placed on ice and irradiated with UV light (10^5 erg/mm^2) by use of a Stratagene UV cross-linker. The RNA was digested by addition of 30 μg of RNase A and incubation for 15 min at 37°C. After addition of loading buffer, the samples were boiled and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography. For the experiments in Figure 2C, competitor RNAs were added to the reaction mixtures immediately after addition of the labeled probes.

DNA Constructions

Deletion and point mutations in the *osk* C region were introduced by polymerase chain reaction (PCR) (details available on request). Nucleotide substitutions in the AB region were introduced by assembling a synthetic EcoRI–HindIII restriction fragment (top strand oligos: AATTCGCTTAGTTTTAATtaGTTTTtaATtgAGatTGTTCTCTGTCTTTG-TTatTTTAgATtTTCGTGCACTT and GTCCTAGTCCATTATTtTAgA-TTATTtTGgGTTTTGgtTTCTtaGTTAGATTTAA; bottom strand oligos: AGCTTTAAATCTAACtaAGAAacCAAAACcCAaAATAATcTAaAATAAT-GGACTAGGACAAGTGCACG and AAaATcTAAAatAACAAAGACAGA-GAACAatCTcaATtaAAAACtaATTAAAACTAAGCG; restriction sites are underlined, and mutated bases are shown in lowercase) from which an EcoRI–DraI fragment was subsequently recovered.

Mutant *osk* transgenes with altered BREs were made in the context of a genomic *osk* DNA fragment previously shown to rescue *osk*⁻ maternal-effect lethality (Kim-Ha et al., 1991). An *exu* transgene carrying BRE sequences was constructed in the context of a DNA fragment previously found to rescue fully the *exu*⁻ maternal-effect defect (Macdonald et al., 1991). A synthetic BRE, essentially two copies of the bruno-binding sites from the *osk* 3′UTR C region (Figure 2), was inserted into the BamHI site positioned near the beginning of the *exu* 3′UTR.

Transgenic Flies

Transgenic flies were generated by P element–mediated transformation (Spradling and Rubin, 1982) using w^{1118} flies as recipients. The P[*osk*⁺] transgenes were those described previously (Smith et al., 1992). For the P[*oskBRE*⁻] transgene, six independent transgenic lines were characterized. Five had similar body patterning phenotypes, while one line with a much stronger phenotype behaved as if it carried two transgene insertions. P[*oskBRE*⁻]-7 carried the transgene on the second chromosome and was used in combination with third chromosome mutants (osk^6/osk^6, nos^{BN}/nos^{BN}, orb^{mel}/orb^{mel}). All of the experiments with *osk* mutants were repeated with osk^6 hemizygotes (using $Df(3R)p^{XT103}$, an *osk* deletion) with similar results. Some of the experiments were repeated using hemizygous osk^1 (a nonsense mutant), again with no substantial differences in the results. P[*oskBRE*⁻]-6 carried the transgene on the third chromosome and was used in combination with second chromosome mutants ($capu^2/capu^2$, $spir^1/spir^1$, $mago^1/Df(2L)F36$, and $stau^2/stau^2$).

RNA and Protein Analysis

In situ hybridizations were performed according to the protocols of Tautz and Pfeifle (1989), with modifications described previously (Kim-Ha et al., 1991). Analyses of *exu* and *rp49* mRNAs were as described (Macdonald et al., 1991; Macdonald and Struhl, 1988). Osk protein was detected in whole-mount ovary preparations as described (Macdonald et al., 1991), by using an antiserum raised against a bacterially produced portion of the osk polypeptide consisting of amino acids 478–606. Exu and vas proteins were detected by Western blot analysis using antibodies that have been described previously (Macdonald et al., 1991; Smith et al., 1992).

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